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Long-Term Estradiol Exposure Is a Direct Mitogen for Insulin/EGF-Primed Endometrial Cells and Drives PTEN Loss-Induced Hyperplasic Growth

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Loss of tumor-suppressor *PTEN* is the most common alteration in endometrial carcinoma. However, the relationship between loss of *PTEN*, growth factors [eg, insulin/insulin-like growth factor (IGF)-1], epidermal growth factor (EGF), and hyperestrogenism in the development of endometrial carcinoma is still controversial. By using three-dimensional (3D) cultures of $PTEN^{+/+}$ and $PTEN^{+/-}$ endometrial epithelial cells, we investigated the effects of EGF, insulin/IGF, and estradiol in endometrial cell proliferation. We have previously demonstrated that 3D cultures of endometrial cells require EGF and insulin/IGF to proliferate. Herein, we demonstrate that, in the presence of EGF and insulin/IGF, long-term estradiol treatment directly induces proliferation of 3D cultures. Moreover, we show that the mitogenic effects of estradiol require the presence of insulin/IGF and EGF, because withdrawal of such factors completely abolishes estradiol-induced proliferation. In the presence of EGF and insulin/IGF, $PTEN^{+/-}$ and $PTEN^{+/+}$ spheroids display a similar rate of proliferation. However, the addition of estradiol causes an exaggerated proliferation of $PTEN^{+/-}$ cultures, leading to formation of complex structures, such as those observed in endometrial hyperplasia or carcinoma. In summary, we demonstrate that EGF and insulin/IGF prime endometrial epithelial cells to direct the mitogenic effects of estradiol. Furthermore, *PTEN* deficiency results in enhanced responsiveness to this combination, leading to the development of hyperplasia of endometrial cells in culture. *(Am J Pathol 2013, 183: 277–287; http://dx.doi.org/10.1016/j.ajpath.2013.03.008)*

17β-Estradiol (E2) is required for epithelial cell proliferation of endometrial epithelial cells. Although it is well known that E2 is a potent mitogen for endometrial epithelial cells, the molecular mechanisms by which E2 triggers its proliferation are still under active research. It has been extensively demonstrated that E2 alone is unable to induce proliferation of endometrial epithelial cells.[1–4] Because E2 itself is not a direct mitogen for epithelial cells in the uterus, different models to explain how E2 induces proliferation have been proposed.[5] The most widely accepted hypothesis is that growth factors, such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and transforming growth factor α, mediate E2-induced proliferation.[6–10] Among them, the role of IGF-1 in mediating E2-induced proliferation has been intensively studied and supported by IGF-1 knockout mice, in which proliferative response to E2 is abolished.[11]

The role of estrogen receptor α (ERα) in mediating E2 proliferation is also controversial. ERα is essential to mediate the proliferative effects of E2.[12] Classic experiments demonstrated that stromal ERα mediates the mitogenic effects of E2 on epithelial cells.[13] It has also been demonstrated that ERα is required for the expression of IGF-1 in the stromal compartment.[14] IGF-1 secreted from stromal cells acts on epithelial cells to mediate the mitogenic effects of E2.[10] More recently,

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ERα ablation on epithelial cells demonstrates that ERα is dispensable for the mitogenic effects of E2 in endometrial epithelial cells.[15] These results contrast with the observation that ERα is required to drive the mitogenic effects of a variety of growth factors, including IGF-1/insulin[16,17] or EGF,[18–22] in endometrium and other tissues.

In the normal uterus, the mitogenic effects of E2 are crucial to complete estrous cycles or to support pregnancy, but excessive E2 exposure is a major risk factor for the development of endometrial carcinoma (EC).[23–25] The most important signaling pathway in the regulation of uterine epithelial cells is the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, and alterations of phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha isoform (PI3KCA) or pentaerythritol tetranitrate (PTEN) lead to development of EC.[23,26,27] PTEN inactivation is an early event in endometrial carcinogenesis,[28–30] and it has been demonstrated that sporadic endometrial mutations in PTEN are frequent in the histologically normal endometrium of women of reproductive age.[4] Therefore, it has been hypothesized that those *PTEN*-null latent precancerous cells progress through endometrial hyperplasia and carcinoma on exposition to risk factors, such as excessive estrogen exposure.[3,4] The role of PTEN in EC is evidenced by *PTEN* knockout mice. Nearly 100% of $PTEN^{+/-}$ female mice develop endometrial hyperplasia, of which approximately 30% progresses to EC.[31,32] The role of PTEN in endometrial neoplastic growth has been further demonstrated by the recent generation of mice with conditional deletion of both *PTEN* alleles.[33] However, the role of ERα in PTEN-driven EC is not fully understood. Although it has been demonstrated that inhibition of ERα expression hampers the development of EC in $PTEN^{+/-}$ mice,[34] a recent work has demonstrated that $PTEN^{+/-}$ $ER\alpha^{-/-}$ mice show an even higher incidence of *in situ* and invasive carcinoma, suggesting that endometrial tumorigenesis can progress in the absence of ERα.[35]

The complex interplay between E2 and growth factors, such as EGF or insulin/IGF-1, to promote endometrial cell proliferation in normal and *PTEN*-deficient endometrial epithelial cells is not completely understood. Herein, by using a three-dimensional (3D) culture of isolated endometrial epithelial cells, we have investigated the effects of the major players in the regulation of endometrial proliferation, which are E2 and the growth factors insulin/IGF-1 and EGF. Finally, we have assessed the effects of PTEN alterations in the response to E2 and growth factors and its role in endometrial development of hyperplasia/carcinoma.

Materials and Methods

Reagents and Antibodies

The recombinant basement membrane Matrigel was purchased from BD Biosciences (San Jose, CA). Epidermal growth factors, ICI182170 (ICI) and LY 294002, were obtained from Sigma (St. Louis, MO); insulin-transferrin-sodium selenite supplement was obtained from Invitrogen (Carlsbad, CA). Antibody to E-cadherin was from BD Biosciences, zonula occludens protein-1 was from Zymed (San Francisco, CA), and bisBenzimide H33342 trihydrochloride (Hoechst), rhodamine-conjugated phalloidin, and antibodies to laminin and tubulin were obtained from Sigma. Alexa-Fluor–conjugated anti-rabbit and anti-mouse antibodies were from Invitrogen. Anti–phophorylated-Akt and phophorylated-extracellular signal–regulated kinase antibodies were from Cell Signaling Technology (Beverly, MA). Anti-ERα antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Jackson ImmunoResearch Europe Ltd (Suffolk, UK). All other reagents were obtained from Sigma, unless otherwise specified.

Animals and Isolation of Endometrial Epithelial Cells

PTEN knockout mice (strain B6.129-$PTEN^{tm1Rps}$) were obtained from the National Cancer Institute (Frederick, MD) mouse repository. The C57BL6 and *PTEN* knockout mice used to isolate endometrial cells were maintained in temperature- and light-controlled conditions and fed ad libitum. The Institutional Animal Care Committee of the IRB-Lleida Institute approved all experimental procedures. The isolation of endometrial epithelial cells was processed as previously described.[36] In brief, uterine horns were dissected from 3- to 4-week-old C57BL6 mice. Uteri were washed with Hank's balanced salt solution (HBSS) and digested with trypsin (Invitrogen). After trypsin digestion, epithelial sheets were squeezed out of the uterine pieces. Epithelial sheets were washed twice with PBS and resuspended in 1 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) supplemented with 1 mmol/L HEPES (Sigma), 1% penicillin/streptomycin (Sigma), and fungizone (Invitrogen) (basal medium). Epithelial sheets were mechanically disrupted in basal medium. Cells were diluted in basal medium containing 2% dextran-coated charcoal-stripped serum (Hyclone, Logan, UT) and plated in culture dishes (BD Falcon, Bredford, MA). Cells were cultured for 24 hours in an incubator at 37°C with saturating humidity and 5% CO_2.

3D Spheroid Cultures

Growth of endometrial epithelial cells in 3D cultures was performed as previously described.[36] Twenty-four hours after plating in plastic, cells were washed with HBSS and incubated with trypsin/EDTA solution (Sigma) for 5 minutes at 37°C. Trypsin activity was stopped by adding DMEM containing 10% fetal bovine serum, and clumps of two to eight cells were obtained. Cells were centrifuged at $18 \times g$ for 3 minutes and diluted in basal medium containing 3% Matrigel to obtain 4×10^4 cell clumps/mL. For

immunofluorescence, cells were seeded in a volume of 40 μL per well in 96-well black plates with a microclear bottom (Greiner Bio-one). For Western blot analysis, cells were placed in a volume of 200 μL in 24-well plates (BD Biosciences). In all cases, 24 hours after plating, medium was replaced by basal medium supplemented with 5 ng/mL EGF and a 1:100 dilution of insulin-transferrin-sodium selenite supplement (Invitrogen) and 3% fresh Matrigel [this medium is referred to as bullous ichthyosiform erythroderma (BIE)]. Medium was replaced every 2 to 3 days.

Immunofluorescence

3D cultures were fixed with formalin for 5 minutes at room temperature, washed twice with PBS. Depending on primary antibody, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes or permeabilized with 100% methanol for 2 minutes. Next, cultures were incubated overnight at 4°C with the indicated dilutions of antibodies: anti-laminin (1:500), rhodamine-conjugated phalloidin (1:500), E-cadherin (1:250), zonula occludens protein-1 (1:250), and anti-GM130 (1:100). After 1 day, cells were washed twice with PBS and incubated with PBS containing 5 μg/mL of Hoechst 33342 and a 1:500 dilution of Alexa Fluor secondary anti-mouse or anti-rabbit antibodies for 2 hours at room temperature. For double-immunofluorescence staining, cells were incubated with the second round of primary and secondary antibodies. In all double-immunofluorescence stains, first and second primary antibodies were from a different isotope. Immunofluorescence staining was visualized and analyzed using confocal microscopy (model FV1000; Olympus, Tokyo, Japan) with the ×10 and the oil-immersion ×60 magnification objectives. Analysis of images was obtained with Fluoview FV100 software (Olympus).

Confocal Imaging and Evaluation of Spheroid Perimeter

Images of endometrial epithelial spheroids were captured and digitized with a confocal microscope (Fluoview FV1000). Epithelial perimeter analysis was processed by image analysis software (ImageJ version 1.46r; NIH, Bethesda, MD), generating binary images of the spheroids as previously described.[37–39] The presence of one lumen was revealed by phalloidin immunostaining. For each experiment, we quantified at least 100 spheroids. Cell polarity of epithelial cells forming spheroid structures was evidenced by double immunostaining, as indicated in each figure.

Western Blot Analysis

Spheroid endometrial 3D cultures stimulated for the indicated periods of time were washed with HBSS and incubated with trypsin/EDTA solution for 5 minutes at 37°C. Incubation with trypsin was done to allow us to separate the spheroid structures from Matrigel. Trypsin activity was stopped by adding DMEM containing 10% fetal bovine serum, and the cells were lysed with lysis buffer [2% SDS and 125 mmol/L Tris-HCL (pH 6.8)]. Relative protein concentrations were determined by loading an 8% acrylamide gel, transferred to polyvinylidene difluoride membranes, and blotted with anti-tubulin antibody. Band density was determined using Quantity One software version 4.5.2 (Bio-Rad, Richmond, CA). Equal amounts of proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Non-specific binding was blocked by incubation with TBST [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.1% Tween-20] containing 5% nonfat milk. Membranes were incubated with the primary antibodies overnight at 4°C, followed by a 1-hour incubation with secondary antibody, 1:10,000, in TBST. Signal was detected with electrochemiluminescence Advance (Amersham-Pharmacia, Buckinghamshire, UK).

RT-PCR and Real-Time PCR

Total RNA was prepared using the Rneasy mini kit (Qiagen, Germantown, MD), according to the manufacturer's protocol. Reverse transcription reactions were performed using 1 μg total RNA with a TaqMan Reverse Transcription Kit from Applied Biosystems. Quantitative real-time PCR detection of gene expression was performed with the ABI Prism 7000 Sequence Detection System using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers and probes for real-time PCR analysis were from Applied Biosystems. Expression Products: (Ccnd1) Mm00432359_m1, c-fos Mm00432359_m1 and glyceraldehyde-3-phosphate dehydrogenase (Gadph) Mm99999915_g1. Relative expression was determined from C_T values, which were normalized to Gadph as the endogenous control. Experiments were performed at least three times, and statistical significance was determined by Student's *t*-test with *P* value.

Bromodeoxyuridine Incorporation

The bromodeoxyuridine protocol was performed as previously described, with minor modifications.[40] 3D cultures were incubated with 3 ng/mL 5-bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) for 15 hours and then fixed with 4% paraformaldehyde. After DNA denaturing with 2 mol/L HCl for 30 minutes and neutralization with 0.1 mol/L $Na_2B_4O_7$ (pH 8.5) for 2 minutes, cells were blocked in PBS solution containing 5% horse serum, 5% fetal bovine serum, 0.2% glycine, and 0.1% Triton X-100 for 1 hour (Sigma-Aldrich). Subsequently, cells were subjected to indirect immunofluorescence with a mouse 1:100 dilution of anti-BrdU monoclonal antibody (Dako, Carpentaria, CA) and Alexa Fluor—conjugated anti-mouse secondary antibody. Nuclei were counterstained with 5 μg/mL Hoechst 33258, and cells were visualized under a confocal microscope. BrdU-positive nuclei were scored and divided by the total

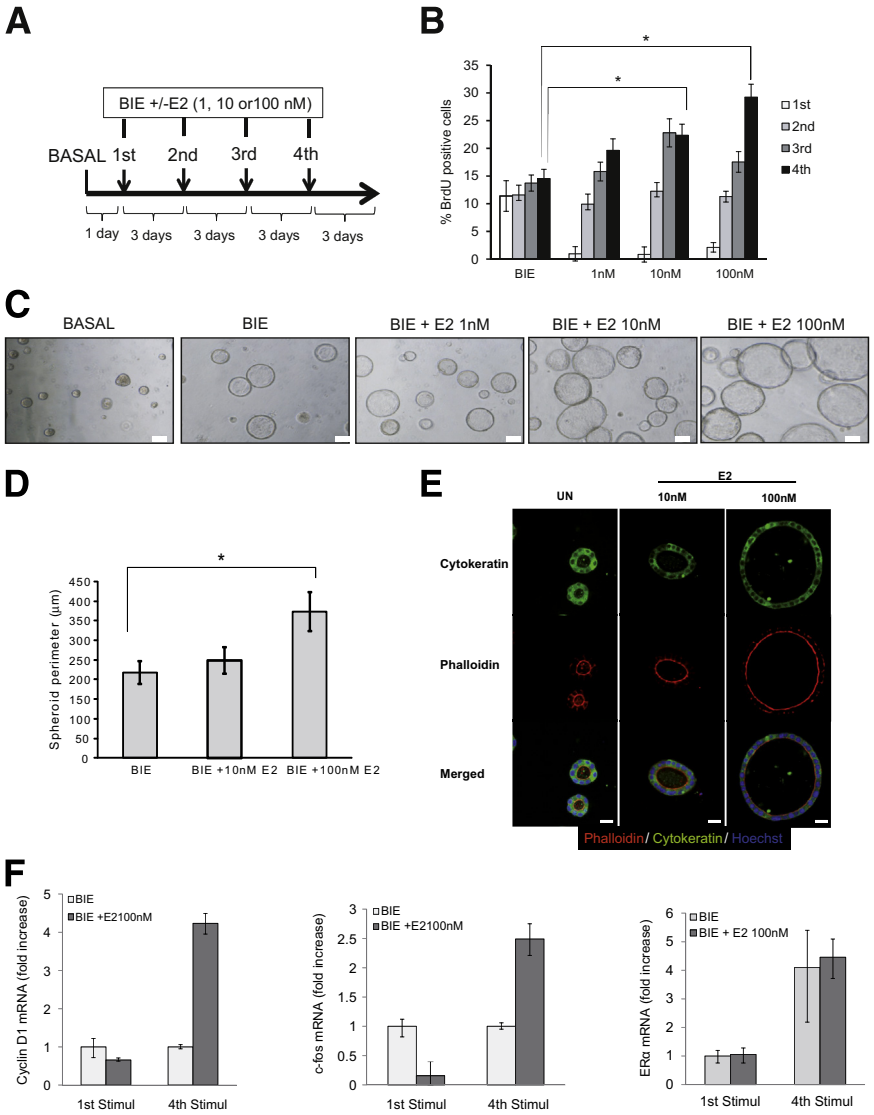

Figure 1 Long-term E2 exposure promotes proliferation of mouse endometrial cells in spheroid cultures. **A:** Diagram showing the protocol used to mimic long-term E2 exposure. Endometrial spheroids were grown for 15 days in the presence of BIE or BIE supplemented with E2 (1, 10, or 100 nmol/L) medium. Medium was replaced every 3 days, four consecutive times. **B:** Quantification of the percentage of BrdU-positive cells after first, second, third, and fourth stimulation with E2. **C:** Micrographs corresponding to representative fields of spheroid cultures grown for 15 days in basal medium (without growth factors), medium supplemented with EGF plus insulin (BIE), or medium BIE plus 1, 10, or 100 nmol/L of E2. **D:** Quantification of gland perimeter of cultures treated during 15 days with BIE or BIE supplemented with 10 or 100 nmol/L E2 medium. **E:** Representative confocal images of phalloidin/cytokeratin immunofluorescence of 3D cultures grown for 15 days in BIE or BIE plus 10 or 100 nmol/L E2 medium. **F:** Relative mRNA expression levels of cyclin D1, c-Fos, and ERα after the indicated stimulations with E2. Results represent means ± SEM. Scale bars: 75 µm (**C** and **E**). $*P < 0.05$, $**P < 0.001$ (Student's *t*-test).

number of cells (visualized by Hoechst staining). The results are expressed as a percentage of BrdU-positive cells.

Statistical Analysis

Experiments were performed at least three times, and statistical significance was determined by the Student's *t*-test.

Results

Long-term E2 Exposure Directly Stimulates Proliferation of Insulin- and EGF-Primed Endometrial Epithelial Cells

Although E2 is essential for endometrial physiological characteristics, hyperestrogenism is a major risk factor for the development of EC.[23–25] It is still unclear whether E2 can induce endometrial proliferation by direct binding to the ERα receptor in epithelial cells. The culture of isolated endometrial epithelial cells as glandular structures (spheroids) provides a suitable scenario to investigate the effects of E2 on epithelial cells, without interference of paracrine factors derived from stromal cells. To mimic a long-term hyperestrogenic situation, spheroids were grown for 12 days in the presence of DMEM/F12 basal medium supplemented with EGF and insulin (BIE) or BIE supplemented with increasing doses of E2 (see protocol for E2 treatments depicted in Figure 1A). Medium was replaced every 3 days for four consecutive times. After four stimulations, we analyzed cell proliferation by BrdU incorporation and spheroid size by measuring its perimeter. The addition of E2 caused a decrease in BrdU incorporation after the first stimulation, but the following treatments progressively increased the number of BrdU-positive cells (Figure 1B). Such an increase was visualized as an increase in the size of spheroids and an increase of the spheroid perimeter (Figure 1, C and D). To demonstrate that E2 increased the size of spheroids but did not cause alterations in its morphological characteristics, we performed double immunofluorescence

with phalloidin (to evidence an apical actin cytoskeleton) and cytokeratin (Figure 1E). To assess whether E2 was able to stimulate transcriptional activity of ERα in epithelial spheroids, we measured the induction of cyclin D1 and c-fos expression by real-time PCR. Consistently, E2 did not increase either cyclin D1 or c-fos expression after the first E2 stimulation, but triggered a marked up-regulation of cyclin D1 and c-fos mRNA after the fourth E2 stimulation (Figure 1F). To demonstrate that ERα was expressed throughout the culture protocol, we performed an RT-PCR analysis of ERα expression. The expression of ERα increased with time in culture (Figure 1F). To exclude the possibility that the increases in spheroid perimeter were caused by increased cellular size, rather than proliferation, we performed individual cell diameter measurements. E2 treatment did not increase cellular size (Supplemental Table S1), indicating that long-term E2 exposure directly stimulates proliferation of mouse epithelial endometrial cells in 3D culture.

Both E2 and growth factors, such as EGF and IGF-1, have been shown to play a pivotal role in the proliferation of endometrial cells, and cross talk between estrogens and growth factors has an important role in cancer development.[17,41] However, the contribution of these factors and their relation in promoting proliferation of uterine endometrial cells is not completely known. Therefore, we investigated whether E2 alone would be able to induce proliferation in cells cultured without EGF or insulin. For this purpose, 3D cultures were grown in the presence of BIE for 7 days to allow formation of spheroids. After 7 days, BIE was replaced for basal medium (without EGF and insulin) containing 10 or 100 nmol/L of E2 (Figure 2A). As a control, matched cultures were maintained with BIE alone or BIE plus E2, 10 or 100 nmol/L. In contrast to cultures grown in BIE, addition to E2 in basal medium did not increase the number of BrdU-incorporating cells (Figure 2B). Consistently, E2 alone did not increase spheroid perimeter (Figure 2C), it did not alter spheroid polarization (Figure 2D), and it did not modify cyclin D1 levels (Figure 2E). These results indicate that, in the absence of EGF and insulin, E2 by itself is unable to induce proliferation of epithelial endometrial spheroid cultures.

EGF and Insulin Do Not Modify Spheroid Morphological Characteristics and Do Not Significantly Increase Proliferation of *PTEN*$^{+/-}$ Cells

A *PTEN* mutation is the most frequent alteration found in type I endometroid EC.[23,26,27,42] To assess the effect of *PTEN* deficiency on E2-induced proliferation of endometrial cells, we performed 3D cultures with endometrial epithelial cells from *PTEN*$^{+/+}$ or *PTEN*$^{+/-}$ mice. For this purpose, we cultured *PTEN*$^{+/+}$ and *PTEN*$^{+/-}$ epithelial cells in the presence

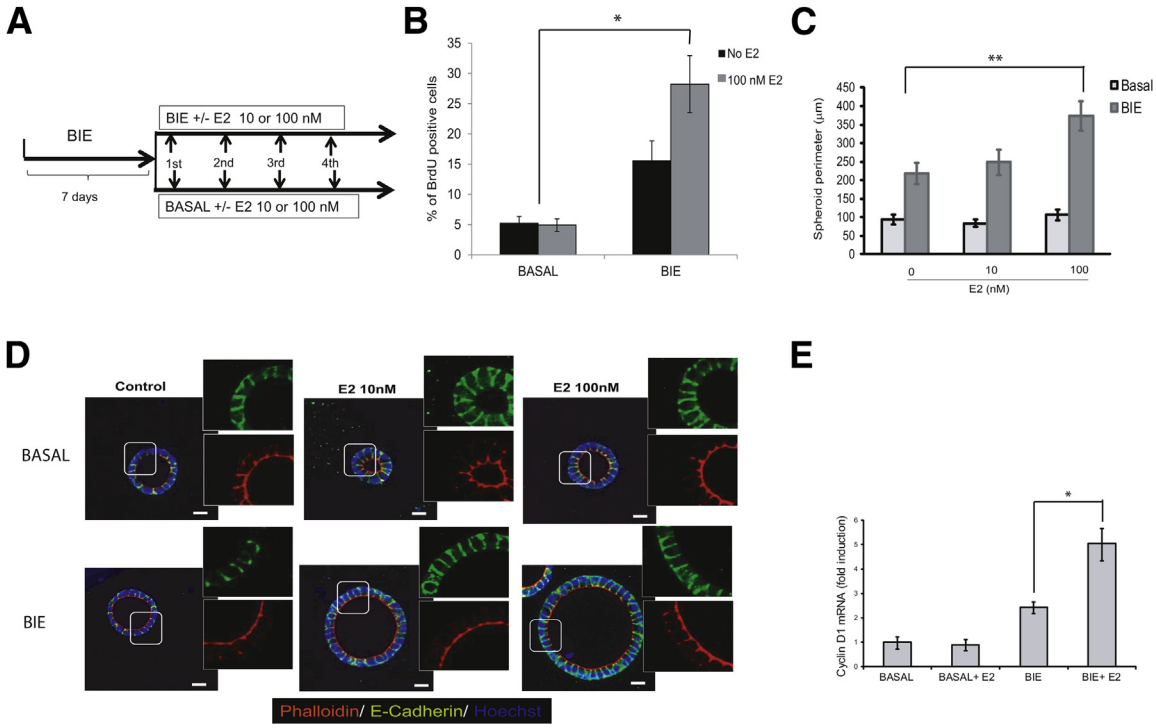

Figure 2 E2 requires insulin and EGF to promote proliferation of endometrial spheroids. **A:** Diagram showing the protocol used for treatment of spheroid cultures with E2 in BIE or basal medium. Endometrial spheroids were grown during 7 days in the presence of BIE medium to allow the formation of correct spheroids. After that period, we grew cells in BIE or basal medium containing 10 or 100 nmol/L of E2. Medium was replaced every 3 days, four consecutive times. **B:** Quantification of the percentage of BrdU-positive cells after four stimulations with BIE or BIE supplemented with 100 nmol/L E2. **C:** Quantification of spheroid perimeter after four stimulations with BIE or BIE supplemented with 100 nmol/L E2. **D:** Representative confocal images of phalloidin/E-cadherin immunofluorescence of 3D cultures grown for 15 days in basal, basal plus 10 or 100 nmol/L E2, BIE, and BIE plus 10 or 100 nmol/L E2. Scale bar = 20 µm. **E:** Relative mRNA expression levels of cyclin D1. Results represent means ± SEM. $*P < 0.05$, $**P < 0.001$ (Student's *t*-test).

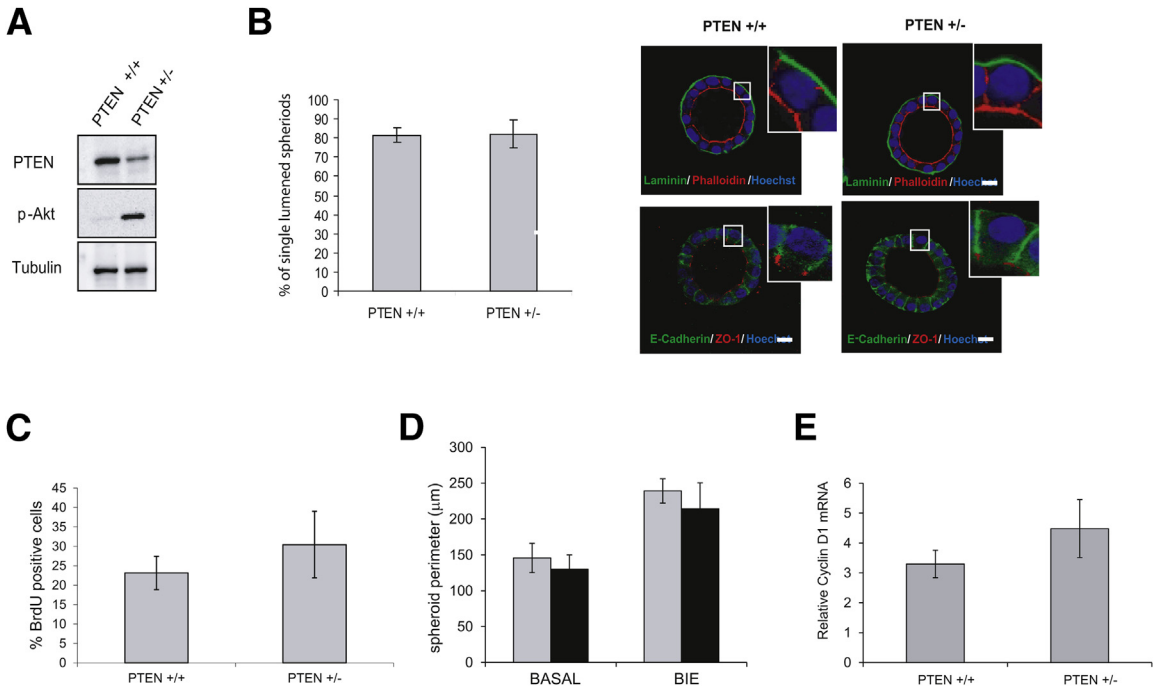

Figure 3 EGF/insulin does not modify spheroid morphological characteristics on *PTEN*$^{+/+}$ and *PTEN*$^{+/-}$ cells. **A:** Endometrial epithelial cells isolated from *PTEN*$^{+/+}$ or *PTEN*$^{+/-}$ mice were grown in 3D for 15 days in BIE medium; p-Akt (Ser473) and PTEN levels were determined by using Western blot analysis (day 15). **B:** Quantification of single-lumen spheroids. Representative confocal images of *PTEN*$^{+/+}$ and *PTEN*$^{+/-}$ spheroids immunostained with laminin/phalloidin or E-cadherin/zonula occludens protein-1. Scale bar = 20 μm. **C:** Quantification of the percentage of BrdU-positive cells after four stimulations with BIE. **D:** Quantification of gland perimeter of *PTEN*$^{+/+}$ (grey bars) and *PTEN*$^{+/-}$ (black bars) cells grown over 14 days in basal or BIE medium. **E:** Relative mRNA expression levels of cyclin D1. Results represent means ± SEM.

of BIE or BIE supplemented with increasing amounts of E2 using the same protocol used in previous experiments (Figure 2A). Because it has been reported that loss of PTEN may impair epithelial cell polarity and lumen formation,[43,44] we first analyzed changes in the spheroid morphological characteristics of *PTEN*$^{+/-}$ cells by immunofluorescence. Despite the fact that *PTEN*$^{+/-}$ spheroids, cultured in BIE, had increased Akt phosphorylation (Figure 3A), they showed completely normal morphological characteristics, with a single lumen, apicobasal polarity, and correct positioning of cell-to-cell and cell-to-matrix junctions (Figure 3B). Next, we analyzed the effects of *PTEN* deficiency using BrdU incorporation. *PTEN*$^{+/-}$ spheroids showed only a slight, but not significant, increase in BrdU incorporation (Figure 3C). This slight increase did not result in a significant increase of spheroid perimeter (Figure 3D). Consistently, under these conditions, *PTEN* deficiency did not cause a significant increase in cyclin D1 expression (Figure 3E).

Loss of *PTEN* Renders Endometrial Epithelial Cells Hypersensitive to E2

Having demonstrated that monoallelic loss of *PTEN* does not significantly increase the proliferation of endometrial spheroids in the presence of EGF and insulin, we investigated the effects of E2 treatment on *PTEN*-deficient cultures. The addition of E2 to *PTEN*$^{+/-}$ cultures caused a dramatic and dose-dependent increase in BrdU incorporation (Figure 4A). Such an increase in cell proliferation resulted in a marked increase in spheroid size of *PTEN*$^{+/-}$ cultures (Figure 4B) and a dramatic increase in spheroid perimeter (Figure 4C). E2 induced an enhanced mitogenic response at 1 and 10 nmol/L. At such doses, E2 was insufficient to cause any significant growth of PTEN wild-type (WT) spheroids. The hyperplasic growth of *PTEN*$^{+/-}$ cultures resulted in an increased complexity of endometrial spheroids (Figure 4D), resembling the morphological characteristics observed in *in vivo* endometrial hyperplasia/carcinoma.

Likewise, in PTEN-proficient cells, withdrawal of insulin and EGF resulted in a complete blockade of E2-induced BrdU incorporation (Figure 4E). Consistently, *PTEN*-deficient cultures treated with E2 in basal medium had no increase in spheroid perimeter (Figure 4F). The dramatic increase of cyclinD1 expression was also inhibited in *PTEN*-deficient cultures treated with E2 in basal medium (Figure 4G). These results demonstrate that *PTEN*-deficient cells require costimulation with EGF and insulin to display hyperplastic growth.

ERα Mediates Mitogenic Effects of EGF/Insulin and E2 in *PTEN* WT and *PTEN*-Deficient Spheroids

EGF and IGF-1 signal through the PI3K/Akt pathway to induce proliferation of both normal and tumoral cell types. Also, ERα mediates the effects of both EGF and IGF-1 in endometrial tissue.[8,16] However, other studies have

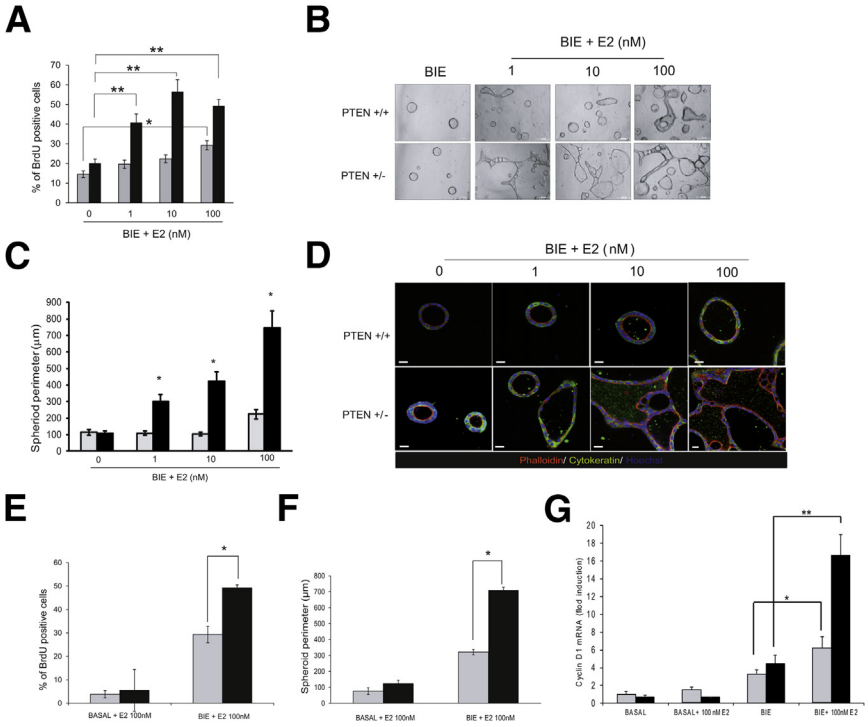

Figure 4 PTEN deficiency renders epithelial cells hypersensitive to mitogenic effects of E2. **A**: Quantification of the percentage of BrdU-positive *PTEN*$^{+/+}$ (grey bars) or *PTEN*$^{+/-}$ (black bars) cells after four stimulations with BIE or BIE supplemented with the indicated doses of E2. **B**: Micrographs corresponding to representative fields of spheroid cultures grown with indicated mediums. **C**: Quantification of gland perimeter of *PTEN*$^{+/+}$ and *PTEN*$^{+/-}$ cells grown in BIE medium or BIE supplemented with indicated doses of E2. **D**: Representative confocal images showing phalloidin/cytokeratin immunofluorescence of 3D cultures grown for 15 days in BIE or BIE plus 1, 10, or 100 nmol/L E2. **E**: Quantification of the percentage of BrdU-positive *PTEN*$^{+/+}$ or *PTEN*$^{+/-}$ cells, after four stimulations with either basal medium or BIE plus 100 nmol/L E2 medium. **F**: Quantification of spheroid perimeter of *PTEN*$^{+/+}$ and *PTEN*$^{+/-}$ cells grown over 14 days in basal plus 100 nmol/L E2 or BIE plus 100 nmol/L E2 medium. **G**: Relative mRNA expression levels of cyclin D1 and c-fos. Results represent means ± SEM. Scale bars: 75 µm (**B**); 20 µm (**D**). $*P < 0.05$, $**P < 0.001$ (Student's *t*-test).

demonstrated that ERα expression in epithelial cells is dispensable for the mitogenic effects of E2.[15] Therefore, we first investigated the contribution of ERα in mediating the proliferative effects of insulin/EGF and E2 in *PTEN* WT cells. For this purpose, 3D cultures were grown in the presence of BIE plus increasing doses of the selective estrogen receptor inhibitor, ICI. The addition of ICI caused a marked decrease in spheroid perimeter (Figure 5A). We further analyzed the induction of cyclin D1 and c-fos as targets of ERα transcriptional activity. BIE increased both c-fos and cyclin D1 expression, which was abrogated by the addition of ICI (Figure 5B). These results suggest that the mitogenic effects of insulin and EGF on endometrial epithelial cells require autonomous expression of ERα. Next, we investigated the role of ERα in the enhancement of epithelial cell proliferation after E2 treatment. The addition of ICI to spheroids cultured in the presence of insulin/EGF plus E2 dramatically reduced spheroid perimeter (Figure 5C). Such a decrease in spheroid perimeter evidences that inhibition of ERα results in a complete blockade of both growth factor (insulin/EGF) and E2-induced proliferation.

Finally, we investigated the role of ERα in mediating the hyperproliferative effects of E2 on *PTEN*$^{+/-}$ cells. The involvement of ERα as a downstream mediator of PTEN is controversial. On one hand, it has been demonstrated that inhibition of ERα expression hampers the development of EC in *PTEN*$^{+/-}$ mice.[34] On the other hand, a recent work has demonstrated that lack of ERα is *PTEN*$^{+/-}$ dispensable for endometrial cancer progression.[35] To address this point, *PTEN*$^{+/+}$ and *PTEN*$^{+/-}$ spheroids were grown for 15 days in BIE plus E2, 100 nmol/L, and the estrogen receptor inhibitor, ICI. Inhibition of ERα expression resulted in a massive decrease in spheroid size (Figure 5, D and E). This result suggests that the effects of *PTEN* deficiency on E2-induced proliferation are mediated by ERα.

PI3K and mTOR Inhibitors Reduce Hyperplasic Growth of E2-Stimulated *PTEN*$^{+/-}$ Spheroids

E2 has been shown to activate the PI3K/Akt signaling pathway to induce cell proliferation in endometrial cells. Therefore, we investigated whether antagonism of the PI3K/AKT pathway would be able to block the profound effect of E2 on proliferation of the *PTEN* $^{+/-}$ cultures. To assess this question, we treated *PTEN*$^{+/-}$ cultures with BIE supplemented with E2 and two inhibitors of the pathway: the PI3K inhibitor, LY294002, and the mammalian target of rapamycin (mTOR) inhibitor, rapamycin. Both inhibitors caused a profound reduction of the *PTEN*$^{+/-}$ spheroid size (Figure 6, A and B), indicating that blocking different elements of PI3K/Akt signaling may be an effective treatment to counteract the hyperproliferative effects caused by E2 on *PTEN*-deficient cells.

Discussion

In the present study, we have investigated the effects of long-term E2 exposure, EGF, and insulin on proliferation of epithelial endometrial cells as a tool to understand their role in the development of endometrial hyperplasia/carcinoma. Both E2 and growth factors have been shown to be important for proliferation of uterine endometrial epithelial

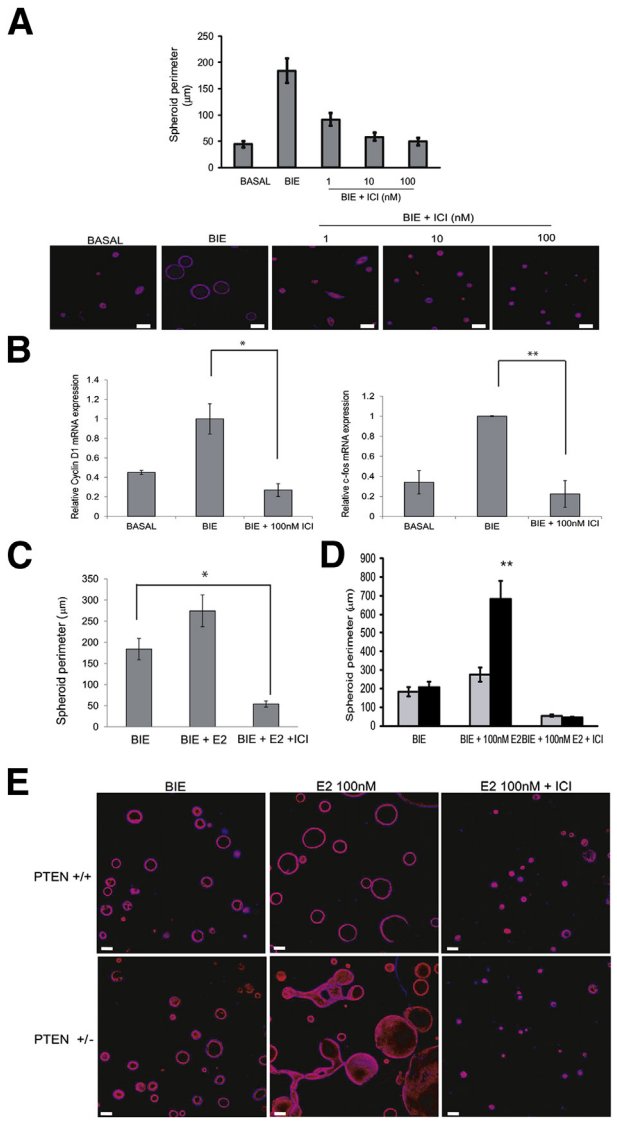

Figure 5 Proliferation promoted by EGF/insulin and E2 in *PTEN* WT and *PTEN*-deficient cells is mediated by ERα. **A:** Quantification of spheroid perimeter after four stimulations with basal medium, BIE, or BIE supplemented with ICI at the indicated dose. Representative confocal images of phalloidin immunofluorescence. Nuclei were stained with Hoechst. **B:** Relative mRNA expression levels of cyclin D1 and c-Fos. **C:** Quantification of spheroid perimeter after four stimulations with indicated mediums. **D:** Quantification of spheroid perimeter of $PTEN^{+/+}$ (grey bars) and $PTEN^{+/-}$ (black bars) cells grown with indicated mediums. **E:** Representative confocal images of phalloidin immunofluorescence of $PTEN^{+/+}$ and $PTEN^{+/-}$ spheroids treated with ICI. Nuclei were stained with Hoechst. Scale bars: 50 µm (**A** and **E**). Results represent means ± SEM. $*P < 0.05$, $**P < 0.001$ (Student's *t*-test).

cells. However, the complex relation between E2 and growth factors, such as EGF and insulin, to promote endometrial cell proliferation is still unclear. Moreover, in the endometrium, the interactions of stromal and epithelial cells add further complexity to the regulation of epithelial cell proliferation. Because of all this complexity, our work has been entirely performed using our recently developed 3D culture of isolated endometrial epithelial cells. This culture allows the development of spheroid structures resembling the endometrial glands found in the endometrium *in vivo*. Because endometrial cells grow in defined medium, our 3D culture provides a simplified method to approach questions that are otherwise difficult to be addressed in a complex *in vivo* model. To this regard, we would like to mention that conditional deletion of loxP-flanked genes in mouse epithelial cells has recently been achieved by the generation of mice expressing *Wnt7a-Cre*[15] or *Sprr2f-Cre*[45] transgenes. There is no doubt that these mouse models will provide more information about the role of different proteins in the regulation of endometrial homeostasis. However, we believe that this culture method can be a valuable tool to study the individual and collective effects of E2 and growth factors on polarized epithelial cells, without interference of paracrine stromal factors.

It is widely accepted that E2 alone is unable to induce proliferation of endometrial epithelial cells.[1,2] Consistent with these results, we have found that E2 alone does not trigger proliferation of isolated epithelial cells, but concomitant presence of EGF and insulin makes epithelial cells sensitive to the proliferative effects of E2. Our findings provide the first evidence that E2 can be a direct mitogen for endometrial epithelial cells. We further demonstrate that the overall proliferative effects of EGF/insulin plus E2 require expression of ERα in epithelial cells. These results seem to contrast with the widely accepted hypothesis that the mitogenic effects of E2 are not mediated by direct action of E2 on ERα of epithelial cells. Classic recombination experiments of Cooke et al[13] demonstrated that the proliferative effects of E2 on epithelial fraction were dependent on the expression of ERα in stromal, but not in epithelial, cells. The authors postulated that engagement of ERα in stromal cells induces the expression of growth factors that are secreted to promote proliferation of epithelial cells. Later, Zhu and Pollard[10] identified IGF-1 as the main factor secreted by E2-primed stromal cells. However, there are several explanations for such discrepancies. First, recent studies have found that ERα knockout used in recombination experiments retains significant ERα activity.[46,47] Second, we demonstrate that a single exposition to E2 is, indeed, unable to induce proliferation, even in the presence of growth factors. Only long-term exposure to E2 renders epithelial cells sensitive to display proliferative effects. Third, the lack of proliferative response in the absence of ERα in stromal cells does not completely exclude direct effects of E2 on epithelial cells. An alternative explanation for these results is that IGF-1 derived from stromal cells sensitizes epithelial cells to the mitogenic effects of E2. Therefore, the lack of ERα in stromal cells would impair IGF-1 secretion, resulting in a lack of response of epithelial cells to E2. Recent studies demonstrate that ERα binding to DNA is required to induce transcription of IGF-1.[48] Moreover, the role of ERα in mediating the proliferative effects of growth factors, including IGF-1 and EGF, has been extensively demonstrated[14,16–22] in endometrium and other tissues.

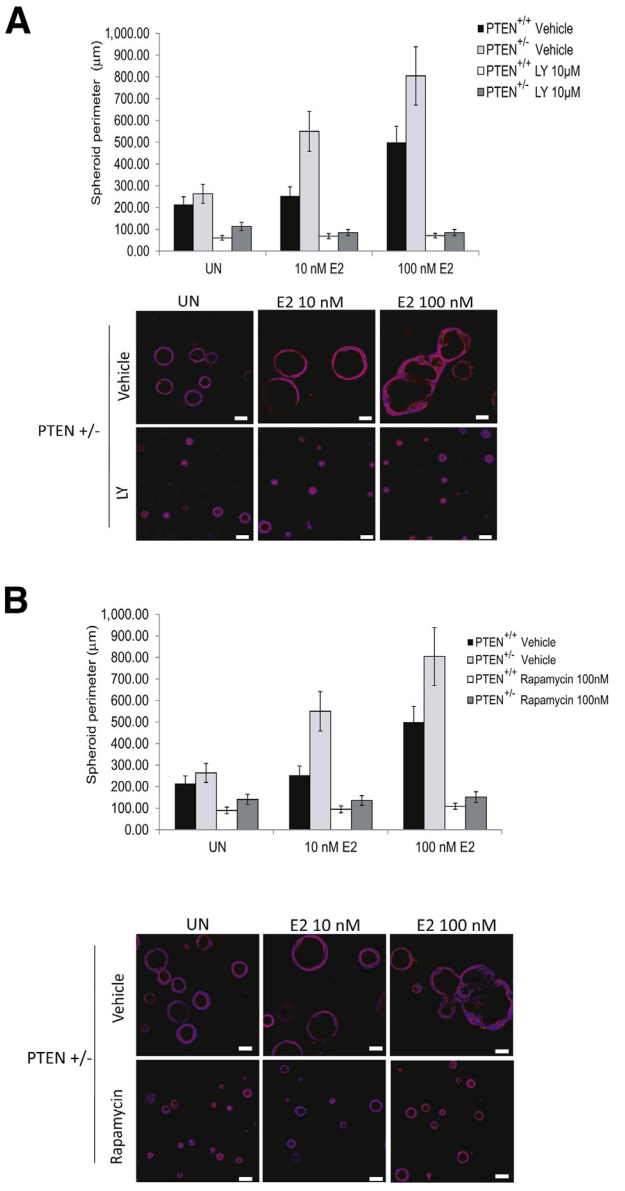

Figure 6 PI3K and mTOR inhibitors block proliferation induced by E2 in *PTEN*$^{+/-}$ spheroids. **A**: Quantification of spheroid perimeter after four stimulations with BIE or BIE plus E2 supplemented with the PI3K inhibitor, LY294002, or its vehicle. Representative confocal images of phalloidin immunofluorescence. Nuclei were stained with Hoechst. **B**: Quantification of spheroid perimeter after four stimulations with BIE or BIE plus E2 supplemented with the mTOR inhibitor, rapamycin, or its vehicle. Representative confocal images of phalloidin immunofluorescence. Nuclei were stained with Hoechst. Scale bars: 10 μm (**A** and **B**).

Recent evidence demonstrates that specific deletion of ERα in epithelial endometrial cells hampers biochemical responses to E2, but it does not compromise proliferation.[15] The authors demonstrate that there is no reduction of DNA synthesis after stimulation with either E2 or IGF-1 in ERα-deficient epithelium. Again, these results may seem at odds with our data, in which we find a decrease in proliferation after inhibition of ERα. However, it has been demonstrated that progression through the G_1 and S phases is normal in *Igf1*-null uterine tissue, showing normal DNA synthesis (and, consequently, BrdU incorporation), but no proliferation. These results indicate that IGF-1 is not essential as a G_1-progression factor. However, *Igf1*-null cells are profoundly retarded in their transit through G_2 and display a dramatic decrease in the mitotic index. Therefore, IGF-1 is required for timely progression through the G_2/M phases of the cell cycle.[11]

We have used insulin as a growth factor for endometrial cells throughout all of the work (as a part of the insulin-transferrin-sodium selenite supplement), but previous studies demonstrate that the main factor involved in mediation of E2 mitogenic effects is IGF-1.[10] Both factors bind to receptors belonging to the IGF-1 receptor family and activate similar intracellular signaling pathways.[49] The basic difference between these two factors resides on their origin: IGF-1 factors can be synthetized in the liver or locally in different tissues (eg, endometrium), whereas insulin is exclusively produced in the pancreas. Obviously, such differences do not exist *in vitro*, and IGF-1 and insulin may cause the same effects in cultured cells.[50,51] To demonstrate that insulin and IGF-1 had exactly the same effects on spheroid cultures, we performed a set of experiments in medium in which we substituted insulin for IGF-1. IGF-1 supported the growth of spheroid cultures and insulin. More important, the addition of E2 in EGF- plus IGF-1–treated cultures resulted in enhancement of spheroid growth ratio similar to that observed in culture grown in BIE (Supplemental Figure S1).

In the second part of our study, we analyzed the effects of long-term E2 exposure on *PTEN*-deficient cells. *PTEN* loss is the most frequent alteration found in EC.[23] However, the factors that influence endometrial carcinogenesis in *PTEN*-deficient cells are still under active research. Recent findings demonstrate that loss of *PTEN* causes disruption of epithelial cell polarity and multiple lumen formation.[43,44,52] Herein, we have found that *PTEN*$^{+/-}$ cells are morphologically indistinguishable from WT spheroids. These results are similar to our previous observations using shRNA to *PTEN*.[53] In this regard, our model provides *in vitro* proof for an *in vivo* model of cancer progression proposed by Mutter and colleagues. These previous series of works demonstrated that loss of *PTEN* expression is an early event in EC progression.[3,29] The authors demonstrated that *PTEN*-null glands are observed in histologically normal endometrium.[3,4] These *PTEN*-deficient glands are morphologically and histologically indistinguishable from normal ones and persist across menstrual cycles. Individual and unremarkable *PTEN*-negative glands become evident when endometrium is exposed to high levels of estrogen. In this scenario, *PTEN*-deficient cells proliferate into dense clusters to form premalignant lesions.

Although we have found that E2, in combination with EGF and insulin, increases the proliferation of *PTEN*$^{+/-}$ cells, the combination of EGF and insulin without E2 did not cause a significant increase in cell proliferation. In contrast, our previous results demonstrated that shRNA to

PTEN produced a slight, but significant, increase in cell proliferation.[36] Such differences can be easily explained by gene dosage, because *PTEN* shRNA causes a higher down-regulation of *PTEN* than monoallelic loss of *PTEN* in hemizygous mice. In contrast, the combination of E2 plus insulin and EGF triggered an exaggerated proliferation that led to the formation of hyperplasic spheroid structures that extraordinary resemble those found in endometrial hyperplasia *in vivo*. Again, our model supports the hypothesis that morphologically normal *PTEN*-deficient spheroids start to proliferate and show features of endometrial hyperplasia after a hyperestrogenic situation.

As previously stated, the involvement of ERα as a downstream mediator of PTEN is controversial. In agreement with the observations of Vilgelm et al,[34] we have shown that ERα inhibition compromises PTEN-induced proliferation triggered by E2. However, we have found a complete inhibition of proliferation induced by E2 plus insulin/EGF and ICI, whereas ICI-treated mice only showed partial reduction of PTEN-induced tumorigenesis. *In vivo*, *PTEN*-deficient cells are exposed to many other factors that can drive endometrial hyperplasia/carcinogenesis independent of ERα. However, our model provides a closer look at the molecular requirements to trigger endometrial proliferation in front to defined factors. E2 also requires the presence of EGF and insulin to enhance proliferation of *PTEN*-deficient cells.

Finally, we have demonstrated that the proliferative effect of E2 on $PTEN^{+/-}$ cells is abrogated by addition of PI3K or mTOR inhibitors. During the past decade, PI3K/mTOR/Akt inhibitors have demonstrated a potential as anticancer drugs.[54,55] Many of such inhibitors are undergoing preclinical evaluation and clinical trials in endometrial cancer.[56] Our results suggest that inhibition of PI3K and mTOR may be effective in cases in which hyperstrogenism led to the development of *PTEN*-deficient endometrial hyperplasia or carcinoma.

In summary, taking advantage of our recently developed spheroid culture of endometrial cells, we studied the effects of E2 and growth factors in normal and *PTEN*-deficient endometrial epithelial cells. We think that our results can be summarized as two main findings: we show, for the first time to our knowledge, that E2 can be a direct mitogen for endometrial epithelial cells, and, we demonstrate that growth factors and E2 cooperate to induce hyperproliferative growth of *PTEN*-deficient endometrial epithelial cells.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.03.008*.

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